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IMPULSE PROPAGATION AND CONTRACTION IN THE TUNIC OF A COMPOUND ASCIDIAN

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ABSTRACT

Diplosoma listerianum and *D. macdonaldi* (Fam. Didemnidae) have a network of cells ("monocytes") in the tunic which contain high concentrations of microfilaments and react positively with NBD-phallacidin, indicating the presence of F-actin. The tunic is contractile, especially in the areas around the cloacal apertures, which can be closed completely. Myocytes are concentrated in sphincter-like bundles around these openings, but also are found throughout the tunic. Electrophysiological recordings reveal a diffuse conduction system in the tunic propagating all-or-none impulses ("tunic potentials," TPs) through all parts with a conduction velocity of $<1.5\ cm \cdot s^{-1}$, and a refractory period of 1.6 s. TPs correlate one-for-one with contractions. The system is excitable to the touch, but is also spontaneously active, showing steady patterns of potentials as well as regular, 'parabolic' bursts. The evidence suggests that the myocyte net itself conducts the impulses triggering the contractions. In the absence of conventional nerves and muscles, the system provides the colony with a way of regulating the effluent water current and hence the volume of a common cloacal space.

The TP system is not 'wired in' to the ascidiozooids either as a sensory or as a motor pathway. The tunic acts as an independent behavioral entity.

INTRODUCTION

The ascidian tunic or test is "an outer covering which completely surrounds the individual zooid in solitary ascidians or forms a common groundwork in which the zooids are embedded in colonial species." (Goodbody, 1974). It is a secretion product of the body wall epithelium and consists of a matrix of proteins and carbohydrates (including cellulose) into which cells migrate from the hemocoel during development. Blood vessels often penetrate the tunic, and sensory processes from receptors whose cell bodies lie in the underlying epithelium may also extend into the tunic (references in Bone and Mackie, 1982) but muscles and nerves[1] are absent. The various cells present may be concerned with secretion of tunic materials, phagocytosis, self-nonself discrimination, coloration, and some other less well understood functions. Some tunic cells are capable of movement and have contractile pseudopodia or filopodia, but the contractions reported are very slow ($<114\ \mu m$ per hour in *Botryllus* according to Izzard, 1974). Several authors (*e.g.*, Saint-Hilaire, 1931; Godeaux, 1964) have likened the tunic to mesenchyme. Brien (1966) calls it "a living envelope, equivalent to a sort of peripheral mesenchyme." Unlike mesenchyme, however, it is not

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[1] There appears to be only one report of nerve cells in the tunic of an ascidian, that of Das (1936). No later study on tunic histology supports this claim.

covered by epithelium but is exposed to the environment, and in this respect it more resembles a cuticular or exoskeletal tissue.

Given the absence of nerves and muscles from the tunic, it is not surprising that there have been no reports that the structure responds to stimulation, contracts, or 'behaves' in the usual sense, although in several cases it is composed of a fairly plastic, viscous material capable of short-term conformational changes (Della Valle, 1908; Godeaux, 1964). During observations on *Diplosoma listerianum*, however, it became clear that this species has a tunic in which electrical signals propagate on an all-or-nothing basis, mediating contractions of the tunic itself. In this report, the electrophysiological characteristics of this conduction system are described, along with an account of the activities performed and of the cells likely responsible for conduction and contraction. The evidence implicates a novel type of cell ("myocyte") as the basis for both conduction and contraction. These cells seem to combine the properties of conventional nerves and muscles including the ability to function as pacemakers. They are distributed throughout the whole tunic in the form of a dense network which, it is proposed, constitutes the structural basis for the behavioral action system whose electrical correlates are picked up with recording electrodes.

Materials and Methods

Two species of *Diplosoma* were used in this study. *D. listerianum* Milne-Edwards, 1841, was obtained at the Stazione Zoologica in Naples, Italy. A species tentatively identified as *D. macdonaldi* Herman, 1886 was obtained at the Friday Harbor Laboratories of the University of Washington, and at the Bamfield Marine Station, Bamfield, British Columbia, Canada. *D. macdonaldi* and *D. listerianum* are very similar and may be conspecific (Monniot, 1974). The specimens collected at Naples grew on the walls of the public display aquarium and elsewhere in the seawater system, where they appear to be endemic. *D. macdonaldi* specimens were collected from rocks and pilings in the intertidal zone. Following the method of Della Valle (1908), specimens were removed from their natural substrates and transferred to glass slides or petri dishes. There they attached after a few hours, subsequently resumed growth, expanded and put out new attachment structures ("crampons"). All the experiments reported in this paper were performed on transplanted specimens maintained in running seawater in the laboratory. The bulk of the work was done at Naples, and *D. listerianum* was used for all the illustrations except Figures 2, 8, and 9.

For histological study, pieces of tunic were dissected out and mounted as whole mounts either fresh or after fixation and examined by phase contrast or Nomarski differential interference contrast microscopy. NBD-phallacidin (from Molecular Probes Inc., 24750 Lawrence Road, Junction City, Oregon 97448) was used as a specific fluorescent stain for F-actin. Material was embedded in Epon 812 for electron microscopy after standard fixation and processing.

Electrophysiological study was carried out on small, whole colonies which had become well established on their glass or plastic substrates. A slow flow of water was maintained through the preparation dish during the experiments to ensure that the colonies behaved as nearly as possible as in nature. Thus, temperatures in the preparation dish were kept close to those in the seawater systems at the laboratories where the animals were maintained (17–19°C at Naples, 11–13°C at Friday Harbor). A simple thermistor flow meter (Mackie *et al.*, 1983) was used to record changes in water flow velocity out of the cloacal apertures. For stimulation and recording, polyethylene suction electrodes were used. Signals were amplified and displayed on an oscilloscope or on a chart recorder. For consistency with our earlier papers on tunicate electro-

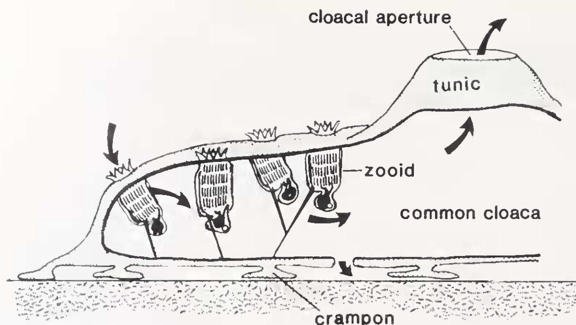

FIGURE 1. *Diplosoma listerianium*, cut-away drawing after Lahille (1890). The zooids hang by their oral siphons from the upper tunic layer and are anchored below by strands of tunic drawn up from the basal tunic layer, which is attached to the substrate by "crampons." Arrows show water flow.

physiology (Bone and Mackie, 1982) the polarity of the electrical records is arranged so that negative events go up, positive down.

General description of Diplosoma *and its activities*

In *Diplosoma* and other didemnids the tunic is drawn out into thin sheets—an upper sheet from which the zooids are suspended and a lower (basal) sheet which attaches to the substrate (Fig. 1). The tunic material composing these sheets is directly exposed to the seawater on both sides, and lacks an epithelial covering. A thin layer of tunic encases the zooids (depicted by Carlisle, 1953) and this continues down into an attachment strand ("stalk") which anchors the zooid to the basal tunic sheet. A retractor muscle and fine blood vessels (30 μm diam.) pass down the stalk from the zooid. It is incorrect to refer to the stalk as the retractor muscle (*e.g.*, Berrill, 1950) as it is composed primarily of tunic, and the muscle penetrates it for only a short distance. The blood vessels entering the stalk, typically four (Pizon, 1905), enter the basal sheet and run out into it, terminating in vascular ampullae. The ampullae contract and expand, pulsating rhythmically as in other ascidians, but never swell to more than 250 μm in diameter. Contrary to the arrangement in colonial styelids such as *Botryllus*, the blood vessels of different zooids are not interconnected. The vascular ampullae are responsible for the formation of 'crampons' (*ramponi, Wurzeln*): specialized patches of tunic material 180–240 μm in diameter by which the basal tunic adheres to the substrate. The ampullae, along with their blood vessels, may withdraw after the crampons are complete, leaving behind an attachment strand of pure tunic material. These strands are most conspicuous around the edges of the colony (Fig. 2). When elongated, they resemble the guy-ropes of a tent (Carlisle, 1961). Crampons are also present underneath the colony, roughly four per zooid stalk.

Water enters the colony through the oral siphons of the ascidiozooids. As the zooids lack atrial siphons, water passes directly out into the common cloacal cavity from which it finally exits via large cloacal apertures, which are often more than 1 mm in diameter. The exhalent water forms a plume that may rise to a considerable height above the surface of the colony. Small apertures (<150 μm) are also present in the basal tunic (Fig. 1) and water passes through them into the narrow space between the tunic and the substrate and then to the exterior. The cloacal apertures are simply holes in the tunic and should not be referred to as siphons, as they are not parts of zooids. A single large cloacal aperture may serve as the exhalent water route

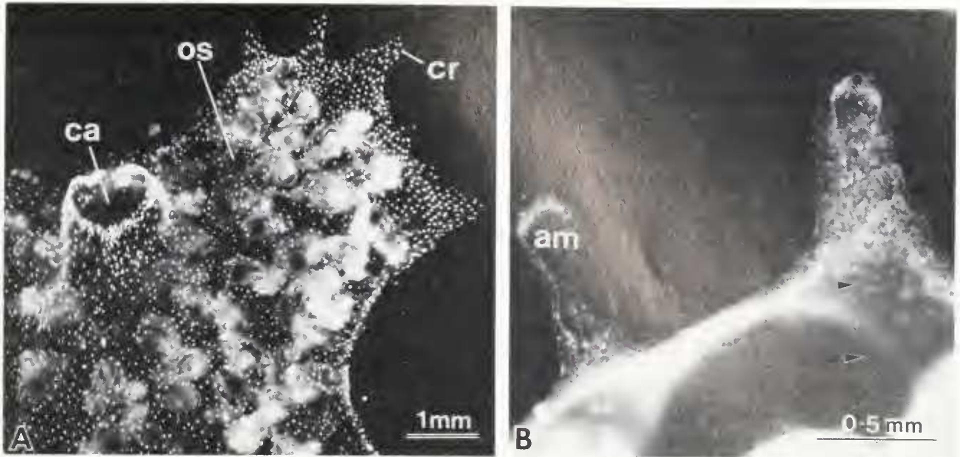

FIGURE 2. *Diplosoma macdonaldi*. A. Portion of a colony seen from above, showing a cloacal aperture (ca), crampons (cr), and zooids, some with their oral siphons (os) in focus. B. Enlargement of edge, showing two crampons, both containing vascular ampullae. The one on the left (am) is expanded, while the one on the right—which comes from another zooid—is contracted. Arrows show the blood vessel of the ampulla on the right.

for some 50 zooids. Stimulation of the tunic at any point results in slow closure of the cloacal apertures, a response discussed in detail below.

A well-maintained colony which is actively feeding and growing in undisturbed conditions tends to be flat, the stalks of its zooids very short (<100 μm), and the common cloacal space relatively small. The blood vessels passing down the zooid stalks extend well out into the basal tunic. Around the edges of the colony these vessels push out and form crampons (Fig. 2B). In colonies which are not feeding and growing so vigorously or which have been kept in stagnant water for a few hours, the blood vessels retract and retreat up the stalks into their zooids, trailing their ampullae behind them. At the same time, the stalks elongate and are drawn out into thin strands 1 mm or more in length. Elongation of the stalks accompanies swelling of the cloacal space with exhalent water, and the whole colony expands. These changes, documented in part by Della Valle (1908, and earlier papers cited), seem to be a response to changed water conditions, but it is interesting to learn that in *Diplosoma virens* expansion and contraction are periodic events exhibited according to a diurnal rhythm (J. S. Ryland, pers. comm.).

Didemnid colonies are known to be capable of locomotion (*e.g.*, Della Valle, 1908; Carlisle, 1961; Ryland *et al.*, 1984). The exact mechanism of locomotion has never been properly analyzed, but it involves the projection of finger-like tunic processes containing blood vessels, whose ampullae form new crampons at attachment sites ahead of the colony in the direction of movement. At the rear end of the moving colony these attachment processes, vacated by their blood vessels, are stretched out thin and eventually detach or break off. There is some evidence of positive phototaxis: Della Valle (1908) found that colonies tended to move upward in the public display tanks at Naples—which are lit from above—stopping only when they reached the surface. Carlisle (1961) found that *Diplosoma* moved sideways when illuminated from the side. Crampons once formed cannot be lifted up and moved to another site, so the movement cannot be thought of as a type of 'walking'; rather, it resembles the

motion of a tracked vehicle, a slow flowing over fixed points which presumably requires secretion of new tunic at the advancing end. The process requires further study.

The ascidiozooids of *Diplosoma* behave like solitary ascidians (reviewed by Bone and Mackie, 1982), pumping water continuously when not disturbed, and contracting their oral siphons and arresting their cilia in response to mechanical interference, as for instance with the entry of an excessively large food particle. Stronger mechanical stimulation causes retraction of the whole zooid by the retractor muscle which runs down into the proximal part of the stalk. These activities are carried on independently by the zooids. Stimulation does not cause the spread of zooid contractions or ciliary arrests across the colony. This is in marked contrast to the situation in *Bortryllus* and its relatives, where signals propagate through the colonial network of blood vessels triggering behavioral events in the zooids (Mackie and Singla, 1983).

Histology

The living tunic is soft, pliable, and transparent. The ground substance shows no regional differentiation except at the surfaces, where there is a thin (50 nm) cuticular layer comparable to the "outermost cuticle" of *Ciona* tadpole larvae (Gianguzza and Dolcemascolo, 1984), but bearing a fuzzy surface coating 200 nm thick. There appears to be no counterpart to the subcuticular zone seen in adults of this and other solitary ascidian species (De Leo *et al.,* 1981; D'Ancona Lunetta, 1983), but a layer about 200 nm deep underlying the cuticle is more densely fibrous than in other regions. Calcareous spicules are present (Carlisle, 1953) but are extremely small (<10 μm) and far apart. Conspicuous in all parts of the tunic are the large, spherical, vacuolated cells termed "kalymmocytes" by Salensky (1892) which are probably the counterparts of the bladder cells (*Blasenzellen*) or Saint-Hilaire (1931) and the *cellules vésiculeuses* of Godeaux (1964). Pérès (1948)—one of the few authors to study postlarval *Diplosoma*—calls them "lacunae," which is clearly inappropriate, as they are cells, not spaces. Also present are cells resembling the granulocytes, morula cells, phagocytes, and other immigrant blood cells described in various tunicates by various authors. Much uncertainty surrounds the identification of such cells, but this is irrelevant to the present discussion. Bacteria are usually present in the tunic ground substance.

Of particular interest in the context of the present investigation are two cell types, both with processes interconnecting to form networks. Neither of these is clearly identifiable on the basis of previous descriptions, so they will be given new names: filopodial cells and myocytes. Filopodial cells (Fig. 3A) are restricted to the surface layer of the tunic, while the myocytes lie deeper. Filopodial cells are flattened in the plane of the surface layer, with three or more broad cytoplasmic expansions resembling neuronal growth cones, each of which subdivides into numerous fine filopodia. The filopodial cells form a fairly regular network, and are spaced out so that the filopodia just make contact. The cells termed myocytes (Fig. 3B, C) are usually bi-, tri- or multipolar, with thicker, much longer processes than the filopodial cells. Their processes show few branches, and rarely subdivide to form filopodia. They are fairly straight, and run for considerable distances through the territories of adjacent myocytes, making numerous contacts with other such processes. The myocyte layer is thick, not two-dimensional like that of the filopodial cells. The myocytes are present in all parts of the tunic but are concentrated into sphincter-like bundles around the cloacal apertures (Fig. 3C) and around the necks of the ascidiozooids. Their presence and circular orientation in these places strongly implicates them in the role of the contractile elements responsible for constricting the cloacal apertures and for pulling

in the tunic over the ascidiozooids when retracted, hence the designation "myocyte." The filopodial cells seem less likely to fulfill such a role, as they show no such concentrations around the openings, and because their processes seem too delicate to be effective as contractile elements.

Material stained with NBD-phallacidin and examined under a fluorescence microscope at 460 nm showed the myocytes as uniformly fluorescent objects, indicating the presence of F-actin (Fig. 4A). Kalymmocytes also reacted positively, but other cells in the tunic showed little response. The filopodial cells showed a very weak fluorescence, and only their thicker processes could be seen at all.

Under the electron microscope (Fig. 5), the myocytes are characterized by dense masses of rather loosely arranged fine microfilaments. True smooth muscle in ascidians by contrast shows thick and thin myofilaments arranged in strictly parallel arrays (Nevitt and Gilly, 1986). Further, using NBD-phallacidin, true muscle from the mantles of the ascidiozooids in *Diplosoma* showed a much stronger fluorescent reaction (Fig. 4B) than was apparent in myocytes in the same preparations. For these reasons, and because of their arrangement in the form of a diffuse plexus, it seems appropriate to recognize the myocytes as a new cell type distinct from conventional smooth muscle.

As noted, the filopodial cells and the myocytes lie in different layers of the tunic, and show few points of contact; therefore, while it is conceivable that the filopodial cells could represent a primitive, neuroid conduction network mediating responses of the myocytes, the likelihood of this seems remote. However, we do not know what function the filopodial cells serve.

Results

The tunic pulse system: basic properties

Colonies growing in good condition on slides sometimes show no electrical activity in the tunic. Usually, however, it is possible to detect spontaneous patterns of small electrical impulses (tunic pulses, TPs) which propagate without decrement through all parts of the tunic, showing no alteration in wave form even when recorded at distances of several centimeters from the site of initiation. They can be conducted through narrow bridges of tunic less than 0.5 mm wide. They are exhibited in newly settled colonies with only two or four zooids. TPs may be evoked by tactile and electrical stimulation of the tunic as well as appearing spontaneously. Their characteristics may be summarized as follows:

Wave form. When the electrode is first attached it may be impossible to detect any signal above the noise level, as a dense plug of tunic must first fill the tip of the electrode. However, once the electrode is well attached, and usually after 30 minutes, signals can be recorded without difficulty for hours or even days. With fine suction electrodes (*ca.*, 50 μm I.D.) attached to the surface of the tunic, the signals are recorded as initially positive-going events rarely exceeding 100 μV in amplitude, with a small but long-lasting negative after-potential (Fig. 6A), and a total duration of about 2.5 s. With larger-bore electrodes the events are more symmetrically biphasic. Recordings from the inner and outer surfaces show similar TP wave forms and amplitudes. Attempts to record intracellularly from the myocytes failed, so interpretation of these extracellularly recorded events is difficult, but they are probably compound action potentials representing the summed depolarizations of many conducting elements. Somehow, the topography of the electrode attachment area converts these summed negative events into a predominantly positive-going signal. With a fine elec-

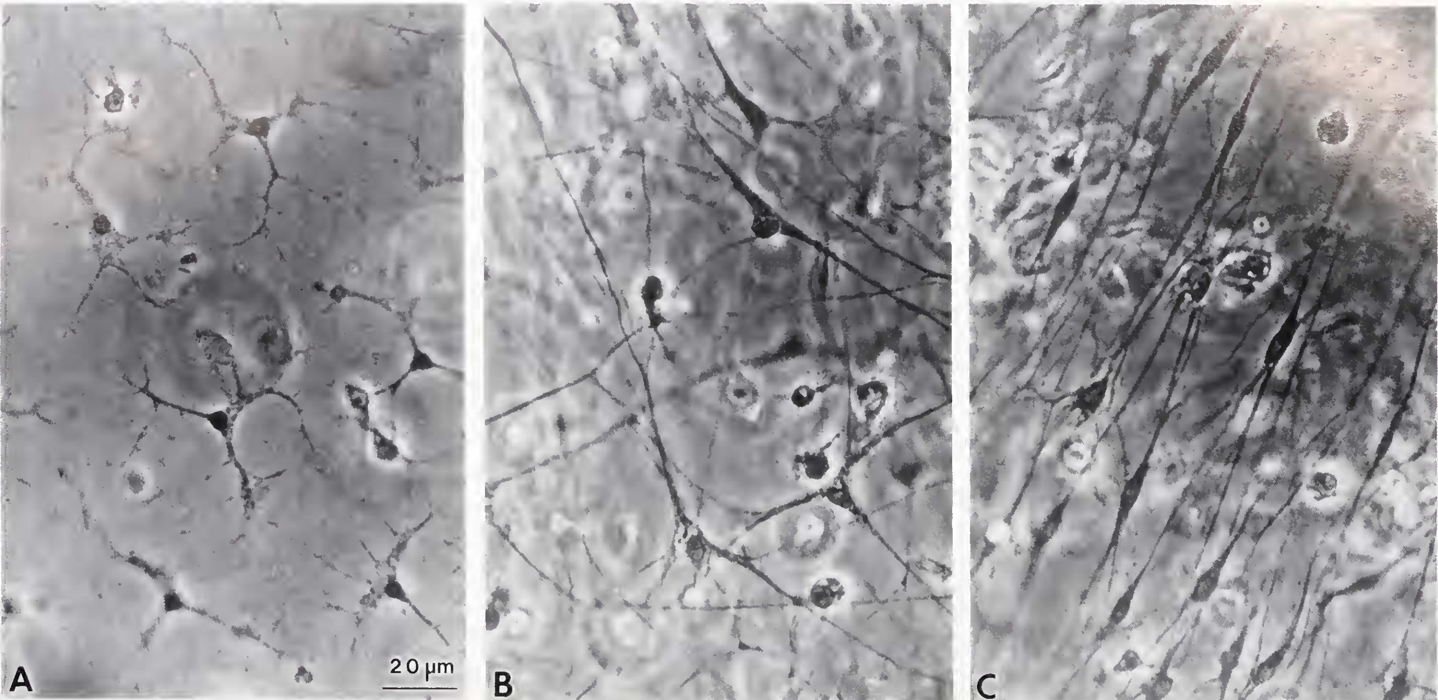

FIGURE 3. Cell networks in the tunic seen by phase contrast, all to the same scale. A. Filopodial cells, in a layer near the surface of the tunic; B. Myocytes from deeper in the tunic; C. Myocytes in circular bundle from edge of cloacal aperture.

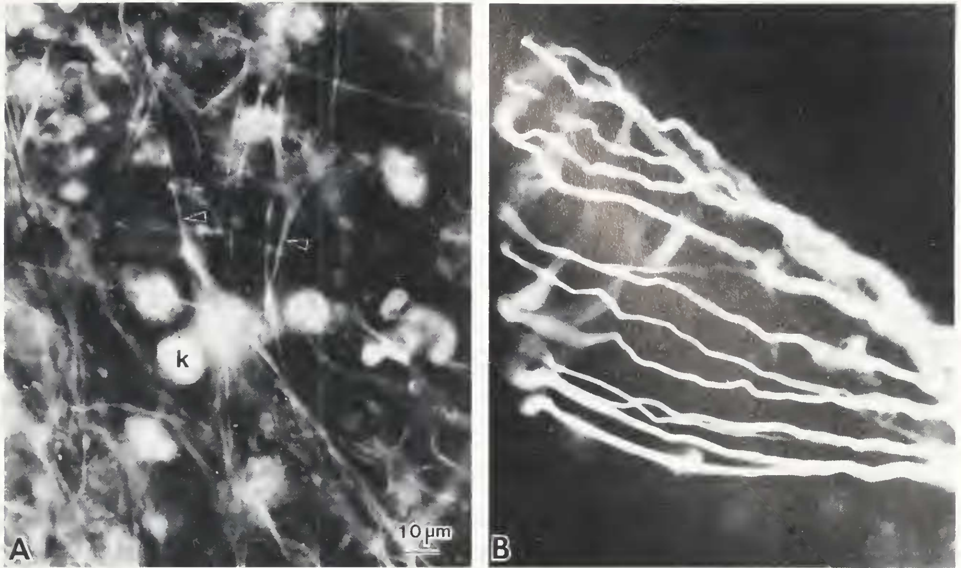

FIGURE 4. NBD-phallacidin: A. Fluorescent reaction in myocyte net (arrowheads), and in shrunken kalymmocytes (k); B. In conventional muscle from mantle wall of a zooid.

trode there would be relatively few conducting elements contributing to the signal, and they would tend to fire in synchrony so the wave form shown in Figure 6A may closely approximate the fundamental event recorded d.c. from a single cell.

Slow conduction. Conduction velocity is 1.0–1.5 $cm \cdot s^{-1}$ at 19°C (Fig. 6B). No

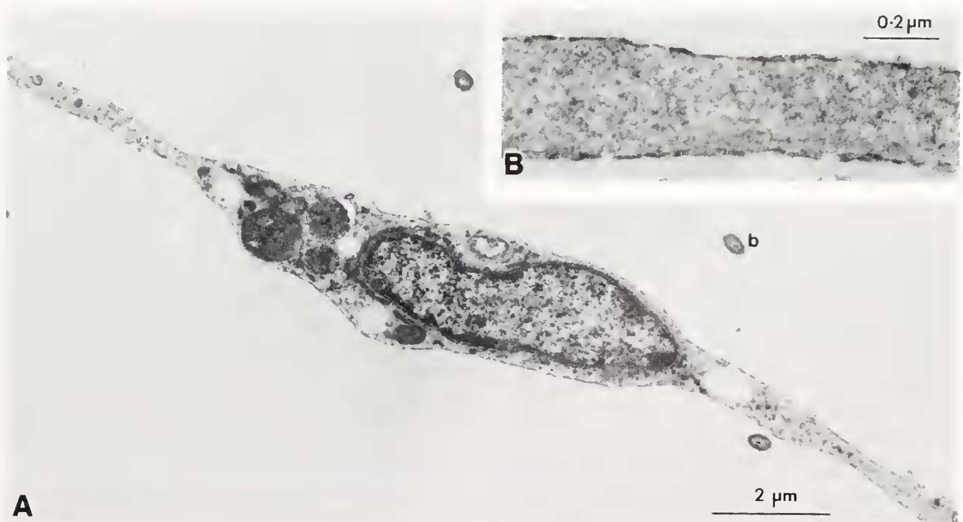

FIGURE 5. Electron micrographs of a myocyte (A) and its process enlarged (B), showing fibrillar contents. Bacteria (b) are often present in the tunic ground substance.

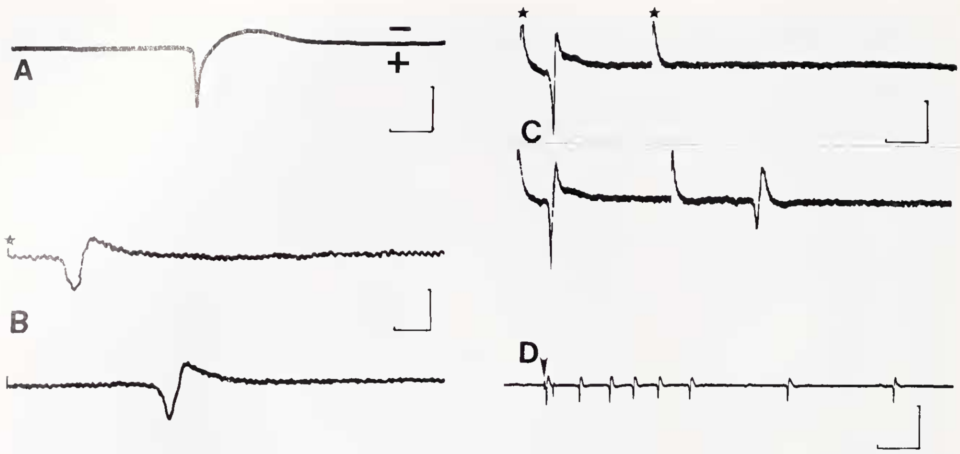

FIGURE 6. Tunic pulses (TPs). A. Spontaneous TP recorded under optimal conditions with a fine (50 μm I.D.) extracellular suction electrode (scale bars: 1 s, 100 μV). B. A TP recorded sequentially from the inside of the basal sheet of the tunic (upper trace) and from the outside of the upper sheet (lower trace) following a shock (*) on the basal sheet. Recording electrodes were 3 mm apart, conduction velocity 1.3 cm $\cdot$ s^{-1} (scale bars: 100 ms, 50 μV). C. With two shocks (*) 1.6 s apart, a response was elicited only to the first shock (upper trace). When the interval between shocks was increased to 1.8 s, both shocks were followed by TPs (scale bars: 0.5 s, 50 μV). D. A mechanical stimulus (arrowhead) elicited a burst of TPs (scale bars: 10 s, 200 μV).

significant variations in conduction velocity were observed in different parts of the tunic. Conduction time increases markedly with successive shocks. With shocks at 7 s intervals, conduction time increased by 50% of its initial value after only 6 pulses. It is not clear if increasing conduction time is due to slower conduction or to passage of impulses via less direct routes.

Long refractory period. At 19°C, the absolute refractory period was 1.6 s (Fig. 6C). In the figure, a second response was obtained with two shocks 1.8 s apart, but the amplitude of the second TP was considerably reduced, and showed a longer latency. A long refractory period would be expected if the action potential has a long duration, as proposed above.

Mechanical and electrical excitability. TPs can be evoked by pinching or pricking the tunic (Fig. 6D) or by delivering electrical shocks through a suction electrode attached to it. As with the recording electrodes, a plug of tissue must fill the tip of the stimulating electrode firmly before experiments can begin. Large shocks are needed, undoubtedly due to current shunting through the aqueous component of the tunic. Responses can usually be obtained with stimulator settings of 30–50 V, 2–5 ms, using an electrode with an internal tip diameter of about 120 μm. Chemical sensitivity was not examined in detail, but the mucus and body fluids of a small keyhole limpet (species undetermined) which is the most obvious predator of *Diplosoma* colonies in the seawater system at the Stazione Zoologica had no effect on spontaneous pulse patterns recorded from the tunic. The TP system does not appear to be affected by changes in light intensity, but this aspect also needs further study.

Spontaneity. Specimens studied in as near to natural conditions as attachment of electrodes would allow showed either (a) absence of all electrical activity, (b) steady, almost metronomic pulse trains going on for periods of hours in some cases, typically with TPs 7–10 seconds apart (Fig. 7A), or (c) bursts of TPs repeated at regular inter-

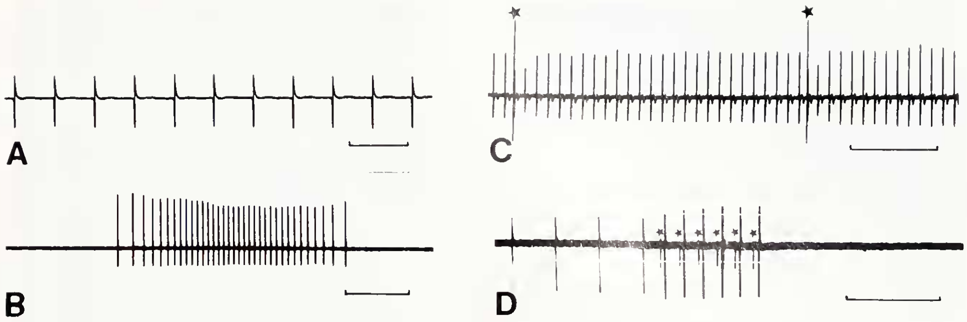

FIGURE 7. Spontaneous TP patterns. A. Steady pulse pattern (scale: 10 s). B. 'Parabolic' burst (scale: 5 min). C. Resetting of steady TP pattern by delivering shocks (*) to produce premature firing of the system. Note that the TP elicited by the shock and the one following are both of reduced amplitude (scale: 1 min.) D. Termination of steady, spontaneous TP pattern by electrical stimulation (*), causing a high frequency, artificial TP burst (6 TPs, 6 seconds apart, scale: 30 s).

vals. These sequences vary considerably, but in typical long-term burst patterns, the bursts last about 10–18 minutes (Fig. 7B), comprise 20–35 individual TPs, and are followed by 10–18 minutes of silence before the next burst. Spike frequency increases during the early part of the burst and decreases toward the end. If spike frequencies are plotted graphically, the curve approximates to a parabola. Parabolic bursting is typical of many pacemakers *e.g.*, many molluscan neurons (Strumwasser, 1968). In a preparation exhibiting a steady TP pattern, delivering a shock slightly before the next predicted spontaneous event resets the pacemaker (Fig. 7C). A steady TP rhythm can be terminated or interrupted by stimulating the preparation at a frequency greater than the rhythm frequency (Fig. 7D).

The ability to produce pulse trains and burst patterns is not restricted to any particular part of the tunic. Small pieces of tunic with no zooids in them from various parts of the upper and basal sheets produced rhythms similar to those seen in intact colonies.

Effect of elevated Mg^{2+}. TP rhythms continued unaffected in 81 m*M* Mg^{2+}. In 105 m*M* Mg^{2+}, spontaneous TP patterns ceased, but the system could still be excited electrically. In 150 m*M* Mg^{2+}, all TP activity ceased. These findings suggest that either conduction, contraction, or junctional transmission in the myocyte network is dependent on extracellular calcium, as magnesium ions block calcium channels (Hagiwara and Takahashi, 1967).

Effect of curare and acetylcholine. Tubocurarine chloride had no effect on the wave forms of TPs nor on their spontaneous patterns when used at concentrations up to 5×10^{-5} $g \cdot ml^{-1}$ over 24 hours. Addition of acetylcholine chloride to the same final concentration had no detectable effect. These findings suggest that nerves are not involved in the tunic responses, as peripheral nerves in tunicates typically operate through cholinergic synapses (*e.g.*, Florey, 1967; Mackie *et al.*, 1974).

Electrical activity of ascidiozooids

Recordings from the zooids show ciliary arrest potentials (CAPs) like those described in numerous other tunicates (reviewed by Bone and Mackie, 1982). As reported by Mackie (1974) for another compound ascidian, *Distaplia occidentalis*, the CAP patterns of different zooids in the colony show no coordination. Attenuated

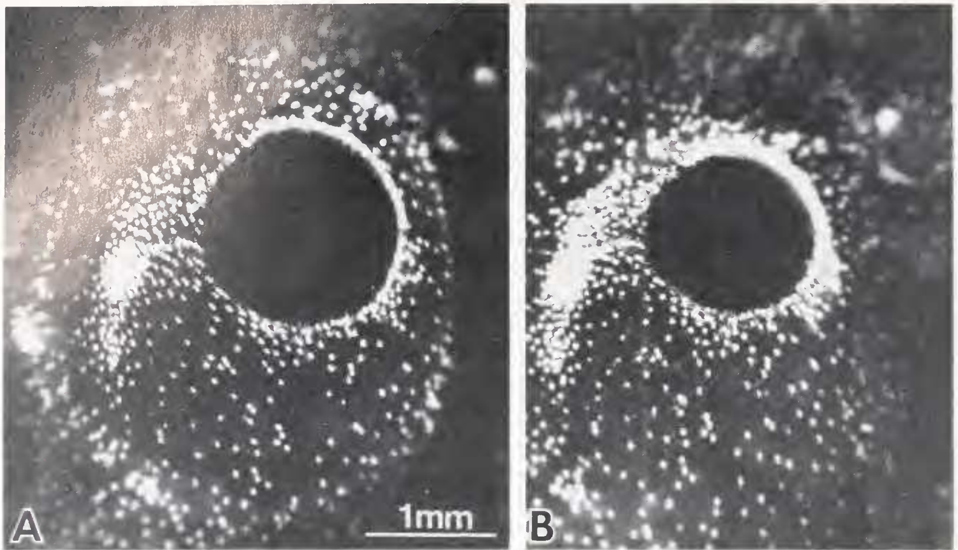

FIGURE 8. Cloacal aperture before (A) and after (B) stimulation of the tunic. Three TPs were elicited 10 seconds apart, leading to reduction of the circumference of the aperture by 17%.

CAPs can be recorded a short distance down the zooid stalk and in the upper sheet of the tunic close to the zooids; these signals are probably picked up electrotonically, rather than being conducted events.

Recordings from the vascular ampullae show small potentials similar to those recorded from the ampullae of colonial styelids and *Perophora*, and like them exhibited in a rhythm coinciding with the contractions which propel blood through the system (Mackie and Singla, 1983). Ampullae belonging to the same zooid are coordinated, but those of different zooids are not. The two ampullae shown in Figure 2B belong to different zooids and are out of phase. Cycle time is about 140 s and, as in *Botrylloides*, the potentials typically occur in doublets.

Effector correlates of tunic pulses

So far as we know, tunic pulses have no relationship to the electrical pulse patterns recorded from the zooids, and vice versa; nor do TPs seem to be involved in the locomotory process. Locomotion has been observed in colonies showing no TP patterns as well as in those showing such patterns. In fact, it seems unlikely that locomotion is controlled by any colony-wide coordinating system. The pulsatile movements of the blood vessels and vascular ampullae certainly play a part in locomotion but they are not coordinated on a colonial basis.

The only clearly demonstrable effect of TP activity is the contraction of the cloacal apertures (Fig. 8). Constriction of the aperture results in an increase in the rate of water flow through the opening. This occurs in a stepped manner, with each step corresponding to a single TP (Fig. 9). Following cessation of TPs, the aperture relaxes slowly. This effect of TPs can be observed both during experimentally induced and spontaneous TP activity, given repetitive firing at a sufficiently high frequency.

For more detailed study, given the sluggish nature of the response, it was conve-

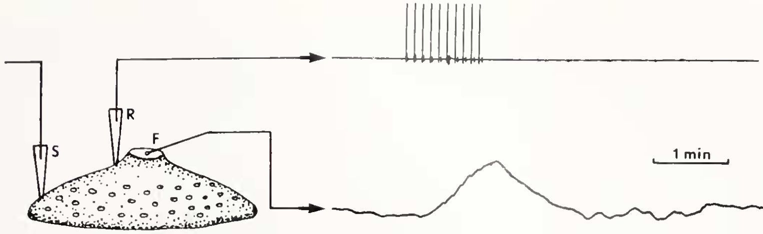

FIGURE 9. Change in rate of water flow through a cloacal aperture following stimulation of the tunic. A stimulating electrode (S) on the tunic evokes TPs, picked up with a recording electrode (R) and shown as small events following large stimulus artifacts on upper trace. Lower trace shows stepped increase in flow rate accompanying the stimulus train, recorded with a glass based thermistor flow meter (F). Following the stimulus train, flow rate returns to normal as the cloacal aperture dilates.

nient to monitor changes in the cloacal apertures visually, using a scalar eyepiece to measure diameters, from which changes in circumference could be calculated. (The myocytes are arranged in circular arrays around the openings, so changes in circumference represent length changes in the contractile tissue.) As expected, long TP bursts produce more contraction than short TP bursts at any given pulse frequency. With shocks set to evoke TPs at intervals of six seconds, summation of contractions is approximately linear until the preparation has shortened to about two-thirds of its resting length, when the curve flattens out (Fig. 10). Pulses more than about 15 seconds apart do not usually produce a summing response. It was observed that relaxation following contraction generally involves a period of hyperextension, after which the preparation returns to its resting length (Fig. 11), but no TP activity accompanies this final phase. Finally, it was shown that with long duration pulse trains at any given frequency, the preparation fails to maintain the level of contraction exhibited initially, but lengthens to a plateau level which is maintained indefinitely (Fig. 12).

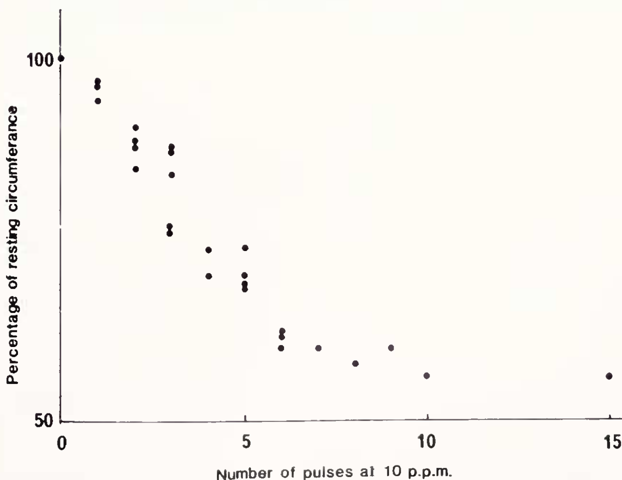

FIGURE 10. Summation of contractions during TP trains evoked by stimulation at 10 pulses per minute.

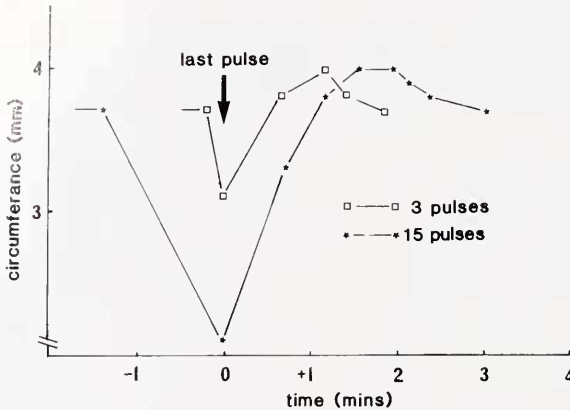

FIGURE 11. Changes in circumference of a cloacal aperture following TP trains of 3 pulses and 15 pulses, both at 10 pulses per minute.

For the experiments reported above, stimulation parameters were deliberately kept at a moderate level, so that each shock produced a single propagated TP. Stronger stimulation which causes multiple firing of the TP system, or repetition of normal stimuli at higher frequencies can produce almost complete closure of the apertures. Under these circumstances, the whole upper surface of the tunic has contracted to some extent, and the cloacal space has diminished. Therefore, although no attempt was made to quantify these observations, it seems clear that the contraction of the cloacal apertures is only part of an overall contractile response involving the whole tunic.

Re-examination of Botryllus

The discovery of a tunic conduction system in *Diplosoma* raised questions about our earlier results with *Perophora* and with *Botryllus* and its relatives (Mackie and

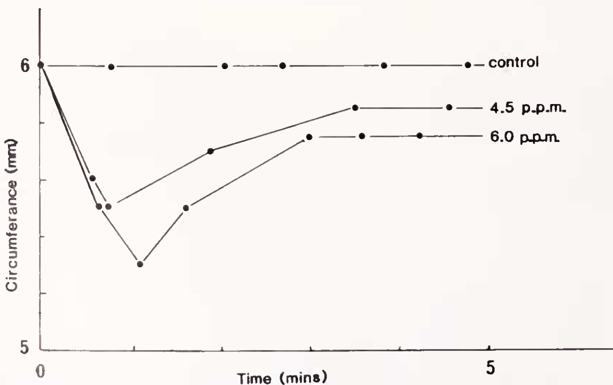

FIGURE 12. Changes in circumference of a cloacal aperture as observed over a five minute period with stimulation at two different frequencies, and with an unstimulated control. Each shock produced a single TP.

Singla, 1983), where we found that coordination of colonial activities occurred by epithelial conduction in the blood vessels connecting the zooids. It is conceivable that in these cases conduction also involves myocytes in the tunic itself. Therefore, the earlier investigation was repeated using *B. schlosseri,* which grows on the walls of the storage tanks at the Naples aquarium. The earlier results were correct. The propagated signals in *Botryllus* can be recorded only from the vascular ampullae and blood vessels. There is no sign of conduction in parts of the tunic where there are no blood vessels. It was confirmed that the blood vessel impulses ("network potentials," NPs) cause ciliary arrests in the zooids, as earlier claimed. Therefore, this NP system in *Botryllus* is distinct from the TP system in *Diplosoma.* It is interesting that *Diplosoma* has a version of the NP system, but it operates only within the confines of individual zooids, and presumably functions to coordinate the contractions of the four vascular branches which run out into the tunic from each zooid. Thus, the NP system occurs in Aplousobranchs (*Diplosoma*), Phlebobranchs (*Perophora*), and Stolidobranchs (various Botryllinae) and must be regarded as a basic ascidian action system. To date the TP system has been identified in only one family of aplousobranchs, the Didemnidae, represented in *Diplosoma,* and may be peculiar to this group.

Discussion

The evidence presented here demonstrates the ability of the tunic of a didemnid ascidian to conduct all-or-none propagated impulses in response to electrical stimulation and for these signals to cause contractions of the tunic. No such findings have been reported for other species, and it seems probable that the properties of conduction and contraction are not widespread in the Ascidiacea, and may indeed prove to exist only in the family Didemnidae. The system enables the colony to control its exhalent water stream, a function performed in most ascidians at the individual zooid level, by muscles in the walls of the atrial siphons. The zooids in didemnids lack atrial siphons, and the only way of controlling water outflow is by regulating the size of the openings in the tunic (the common cloacal apertures). Therefore it seems possible that the properties of conduction and contraction in the didemnid tunic evolved in parallel with the reduction and loss of the atrial siphons of the zooids, primarily as a way of allowing the organism to control its exhalent water currents.

It is not clear exactly what benefits would be associated with the ability to regulate water flow through the colony. Strong stimulation can produce almost complete closure of the cloacal apertures, which might be advantageous in the presence of a predator. Less strong stimulation causes constriction of the apertures and produces narrow, high velocity water plumes, which rise to a greater height above the colony; this would reduce the amount of water recycled through the colony and increase advection of fresh water from the surroundings. Contractility also allows the colony to regulate the volume of water in its cloacal cavity thereby enabling it to expand or contract, an adaptation that might be put to a variety of uses. As noted earlier, *Diplosoma virens,* which possesses photosynthetic symbionts (*Prochloron*) in its tunic, expands and contracts on a diurnal basis (J. S. Ryland, pers. comm.).

We have searched in vain for evidence that the tunic conduction pathway mediates protective responses of the zooids. The majority of colonial animals have some means of coordinating their defensive responses, and this is true of ascidian colonies like *Perophora* and *Botryllus,* whose zooids are coordinated by signals transmitted through the colonial vascular system (Mackie and Singla, 1983). But *Diplosoma* appears to be an exception. Here there is no colonial vascular network and impulses propagated in the tunic conduction system seem to have no effect on the zooids.

Indeed, as Della Valle (1908) remarked in the context of locomotory behavior, the tunic has its own 'individuality,' meaning that it has a life of its own, functioning without reference to the zooids contained in it.

Regarding the cellular basis for conduction and contraction in the tunic, there can be little doubt that the cells termed myocytes are responsible for the contractions. It also seems likely that these cells conduct the electrical signals for their own contractions. The only other cells arranged in a net-like configuration—the filopodial cells—lie in a different layer of the tunic and make few contacts with the myocytes, so they are probably not the conducting elements. There is nothing inherently unlikely in the idea of a primitive contractile system which conducts its own impulses. Vertebrate cardiac muscle and many sorts of smooth muscle show this ability. However, we are hestitant to call the cells in question muscle cells because they exhibit a lower level of differentiation than true smooth muscle cells in tunicates, both in terms of their general morphology and of their ultrastructure. The term 'myocyte' seems best for these actin-loaded cells which lack thick myofilaments, are arranged in a loose network, conduct impulses very slowly, and show very long contraction latencies.

Non-muscle contractility is well developed in ascidians. Tail resorption in ascidian tadpoles involves the rapid transformation of squamous epithelial cells into tall, flask-shaped cells during which actin microfilaments become aligned in the apical (*Distaplia*) or basal (*Botryllus*) cytoplasm. Discussing these findings, Cloney (1982) states that "the caudal epidermis clearly provides the driving force in tail resorption." Sperm release in *Ciona* involves contraction of the sperm duct epithelium, again by organization of actin microfilaments. The assembly of the filaments is triggered by light (Woollacott and Porter, 1977). Microfilaments are also involved in the contractions of the vascular ampullae of colonial styelids like *Botryllus, Botrylloides,* and *Metandrocarpa* (DeSanto and Dudley, 1969; Katow and Watanabe, 1978; Mackie and Singla, 1983). The epithelial cells in these cases communicate via gap junctions, which presumably provide intercellular pathways for transmission of the impulses which coordinate the contractions of the ampullae. A similar mechanism may apply to the myocyte network in the tunic of *Diplosoma,* but intracellular recordings and demonstration of coupling between myocytes are required to prove this. The possibility that the myocytes communicate via chemical synapses cannot be ruled out, especially in view of the system's sensitivity to magnesium.

Thus we believe the tunic myocyte net is a system evolved *de novo* in *Diplosoma* and probably in other didemnids to bring about coordinated contractions of the exhalent water openings, thus bringing water flow under colonial control. Contractions are slow, conduction velocity is the slowest on record for any animal with the exception of hexactinellid sponges (Mackie *et al.,* 1983), the system has a limited carrying capacity in terms of impulse frequency, and it appears to fatigue very quickly. Nerves and muscles probably would allow the animal to respond with much more alacrity; however, there are no nerves or muscles in the tunic of any ascidian, so it would seem that there was no evolutionary starting point for a conventional neuro-muscular system and a completely new type of cell—the myocyte—had to be evolved, albeit as a rather inefficient substitute.

These findings emphasize the unusual versatility of the Tunicata in developing mechanisms of colonial coordination without ever using simple, direct nervous interconnections. *Diplosoma* uses an excitable myocyte network in the tunic, *Botryllus* uses its excitable vascular epithelium, the zooids in a colony of *Pyrosoma* signal to each other by responding visually to each others' bioluminescent flashes (Mackie and Bone, 1978), and salps relay signals between zooids by excitable epithelial pathways arranged in series with nerves (Bone *et al.,* 1980; Anderson and Bone, 1980). It is unlikely that these examples exhaust the list of possible mechanisms.

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